Sucsessful Peripheral Nerve Homotransplantation by Use of High-Voltage Electron Irradiation

AUTHOR(S):

IKEDA, KIMIYUKI

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by

KIMIYUKI IKEDA

From the Department of Neurosurgery, Kyoto University Medical School
(Director : Prof. Dr. Hajime Handa)

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HYPOCRATES had an occasion to note the fact that the nerve had no potential power to repair itself if it had deen once damaged, saying, "When nervous system is cut, it does not repair itself neither does it reunite". Repairing large defects in the peripheral nerves caused by injury has been one of the most vexing problems in the surgical treatment of the extremities. It is doubtless that morphological tissue continuity can be established across such gaps by outgrowth from the stumps without any surgical intervention, but functional recovery after such unaided union can not always be expected. Where end-to-end suture is not possible, the only rational alternative is to apply some form of nerve graft. Since the classical experiment of PHILLIPEAUX and VUIPIAN[29], in which they showed that a grafted nerve might help new nerve fibers grow across the gaps, many authors[2)5)6)10)31)32)] have shown that grafted pieces of nerve could be used to conduct new fibers across the gaps caused by lesions in the peripheral nerves, and various types of nerve graft have been devised and attempted. However, an important problem of repairing large irreducible defects in the peripheral nerves has been left unsolved up to date, although many attempts have been made to prepare grafts hoping to repair all types of the damaged nerves.

Various types of grafts have been tried extensively on the human beings as well as on experimental animals. Transplantation of heterografts (transplanting pieces of the nervous tissue obtained from one species to a different one) has been always failed. Such a graft takes a role of nothing but an irritating foreign body, failing to become fused with the host, and the graft is eventually absorbed without functioning against expectation. No single instance is found in the literatures, in which heterotransplantation of nerve grafts has ever been performed successfully. Though homografts (transplating pieces of the nerve from another individual of the same species) provoke a brisk cellular reaction, they are still capable of conducting outgrowing axons to the periphery. Encouraging results have been obtained with homografts among animals, although they are not entirely satisfactory. Here, discrepancy between the experimental results and their effectiveness when they are applied clinically must be comprehended correctly. Results of homografting applied on the human beings without any particular preoperative treatment have been always quite discouraging.

KLINE and his associates[15)16)], demonstrating differences of species in responses to peripheral nerve injury, stressed the importance of testing the new techniques of peripheral nerve repair on higher primates before attempting them on the human beings. Many investigators[23)24)] have given up an attempt to apply nerve homografs on the human beings for the reasons that the grafts immediately provoke severe foreign-body reaction, degenera-

ting into mere fibrous bands which become collagenous as time elapses, and almost nothing remains of the original nerve elements after a short while and, in addition, there is no definite sign or even possibility of regeneration at any time postoperatively. Further investigations of immune reactions and improved treatments are naturally required aiming the clinical success of nerve homotransplantation[33]. As far as the autografting technique (transplanting pieces of the nerve from the same individual) is concerned, many authors have reported cases of successful recovery of sensation as well as of motor function both in animals and in the human beings. In many cases, however, autograft is not possible if the affected damage of the injured nerve is so extensive as the patient cannot donate sufficient graft to cover the defect without causing considerable neurological damages. Therefore, this method can be applied only in limited cases. MATSON et al.[23] succeeded in experimental axon regeneration by interposing aligning filaments in the defects of the cut nerves. However, any of the intermediate substances such as strands of catgut or silk, multiple threads of nylon or tantalum, and intermediate segments of arteries and veins are considered to be of no use for better results nowadays.

MEDAWAR[24][25] has pointed out a phenomenon of acquired immunity in cases of homotransplantation of the skin and other tissues. According to MEDAWAR, the whole reaction of the host to the grafted foreign tissue is of a type of actively acquired immune reaction and it needs some time to acquire the immunity that provokes the cellular reactions in cases of homotransplantation. Therefore, if a short graft has been used to connect a gap in the nerve, the graft can work successfully as a bridge for the outgrowing axons, and help them reach the peripheral stump, securing a biologically independent environment before the immune reaction destroys the graft. From the clinical standpoint, however, there are seldom cases in which a short homologous nerve graft is indicated. If longer grafts are used, a storm of reaction breaks the grafts befor the outgrowing axons may have arrived at the other side. This is precisely the reason why no cases of clinical application of the nerve homografting with success have been reported.

MARMOR[20][21] reported successful cases of homotransplantation applied on experimental animals and the human beings with use of high-voltage cathode irradiation for the purpose of sterilization together with the technique of freezing the homologous nerve graft in order to increase the host tolerance. He really established the feasibility of utilizing homografts of the sciatic nerve treated by high intensity irradiation and deep freezing. He found in mongrel dogs that gaps up to 3 cm in length could be bridged by the homografted nerve tissue with functional recovery and histological regeneration sufficient to overcome a foot-drop. Later, he reported two cases of functional recovery by grafting irradiated human and calf nerves to dogs[22].

A comparative study made in our laboratory on the immune suppressive effects of high-voltage cathode ray irradiation, boiling, alcohol- or formalin-fixation, of the grafts, local or general administration of adrenocortical hormones and general administration of antimetabolites, revealed that the nerve homografts irradiated prior to implantation reduced most effectively the inflammatory foreign-body reaction generated in rats and dogs. Histologically, minimum cellular reactions were noted in and about the transplanted nerve with homografts with preoperative irradiation. The details of the experiment were reported

at the 23rd and 24th annual meeting of the Japan Neurosurgical Society[12][13] and at the 1st annual meeting of the Japan Society for Transplantation[7].

Present report deals with the abstract of the preliminary experiments, and histological and electrophysiological study of peripheral nerve regeneration by use of high-voltage cathode ray irradiation in dogs. Two clinical cases in which the similar technique was used for the human beings will also be discussed.

MATERIALS AND METHODS

RATS

Surgically removed segments of the sciatic nerve and/or the brachial nerve of either WISTER or SPRAGDAULEY rats were immediately placed and sealed in an ampulla with sterile technique, and were preserved at $-25\sim-30$ degrees centigrade for $24\sim48$ hours, which were then irradiated with 2 million roentgen-equivalent-physicals (REP) for 13.5 seconds with the dose-rate of 150,000 REP per second, at the temperature of dry-ice-alcohol with a VAN DE GRAAFF pressure-insurated electrostatic generator. The grafts were then kept frozen in a deep freeze stocker until being used[33].

WISTER rats were used as recipients to test transplantability of the irradiated nerve homografts. A longitudinal median incision was made on the back of each rat at the level of lumbar spines and a nerve graft was implanted subcutaneously in the frank. Nerve grafts were placed possibly afar from the skin incision to prevent them from being adherent to the operative scar.

Two different types of nerve grafting without irradiation were attempted in the same fashion using the similar technique for comparison. In the first group of control, fresh nerve graftings without any preoperative treatment were used. 3 mg/kg predonisolone, 3 mg/kg 6-mercaptopurine, 3 γ/kg actinomycine-C and $0.3\sim3$ mg/kg methotrexate were generally administrated in some cases of this group. In the second control group, grafted nerves were treated preoperatively in the following fashions; a) grafted nerves were boiled in hot water either at 100°C for 10 minutes or at 60°C for 30 minutes, b) the specimens were fixed in 70% alcohol or 10% formalin for about 1 week, c) the grafts were soaked in solution of adrenocortical hormones, viz. cortisone, predonisolone and dexamethasone for approximately an hour.

The experimental animals were sacrificed 2, 4, and 6 weeks after the operation, and the careful examination was made at the sites of the operation. After gross inspection in and about the grafts, the tissues were removed en bloc and fixed in formalin. Routine histological study was performed on each specimen stained by hematoxylin and eosin. Myelin stain of luxol-fast-blue and the HOLMES' stain for myelin and axons were attempted as indicated.

DOGS

Portions of peroneal nerves and segments of the femoral arteries were surgically taken out with sterile technique from mongrel dogs and were preserved and irradiated in the same way as previously described. Recipient dogs of 75 in number weighing $8\sim15$ kg were anesthetized individually with intravenous nembutal administration. A skin incision

Fig. 1. Method of experiment.

MATERIAL :

DOG·····N. fibularis

↓ −25～−30°C

ELECTRON BEAM IRRADIATION :

200×10^4 REP.

in Dry-Ice-Alcohol Bath

by Van de Graaff Generator

↓ −25～−30°C

TRANSPLANTATION :

DOG·····Homologous Nerve Grafting

of 10 cm in lenght was made on the lateral aspect of the right knee joint. The peroneal nerve was then explored and atraumatically mobilized. Retraction usually produced a defect of 3～4 cm in length after surgical romoval of a section of the peroneal nerve measuring 2～3 cm in length. Implantation of the previously prepared graft was then attempted to fill up the defect. Nerve graft was sutured in place of the defect mostly by tubulation technique with an arterial tube, and partly by 5-0 atraumatic black silk sutures through the nerve sheath. A portion of femoral artery which had been preserved in the deep freezer after irradiation was hereby utilized as the arterial tube during the process of transplantation. Two interrupted sutures of 5-0 black silk or 4-0 nylon were placed between each end of the arterial tube and the epineurium of the nerve (Fig. 1). Fresh nerve without any specific treatment was grafted for control in five cases by tubulation technique immediately after removal from the donor dogs. Antimetabolites were administrated to the homografted dogs with irradiated nerve grafts for the purpose of suppressing the fibrotic proccess which, otherwise, might have occurred from the cut ends of the damaged nerves. For the period of fortnight, 3 mg/kg of 6-mercaptopurine, 3 γ/kg of actinomycin-C and 0.3～3 mg/kg of methotrexate were administrated postoperatively to 13 dogs, 8 dogs and 8 dogs respectively.

Periodical examinations of the graft with care by surgical exploration were performed in a month of operation, approximately 2 months and more than 6 months after the transplantation. Particular attention was paid to inspect how grafted nerve segments, arterial tubes and sites of repair looked like. The amount of scar tissue accumulated around the graft was also examined and recorded. A long segment of the peroneal nerve including the grafted portion in between was excised at approximately 3～4 cm proximal and distal to the grafted margines from most of the experimental dogs. Each excised segment was then carefully studied histologically at different levels. Fourteen dogs were kept alive for more than 6 months postoperatively including one successful case of 175 days survival (Table 1). These dogs for long term observation were finally explored with intravenous nembutal anesthesia. Sufficient airway was secured by intratracheal intubation for further experiments under anesthesia. Every one of the available electrophysiological examinations

Table 1. Long term observation of dog nerve homografting.

Dog No.	Length of graft cm	Interval of observation days	Conductivity
8	4.0	207	+
16	3.5	196	−
17	3.5	203	+
22	3.5	180	+
23	4.0	194	+
24	4.0	208	+
25	3.5	202	+
26	4.0	209	+
27	4.5	203	+
28	3.5	206	+
34	4.0	341	−
35	4.0	334	+
38	4.5	322	+
39	3.5	175	+

[18)27)] was attempted to 12 dogs out of 14 observed for long duration. In two cases, unfortunately, the grafts were found to have been separated from the distal stumps at the time of exploration. In the first place, functional recovery of the dorsiflexor muscle of the foot innervated by the peroneal nerve was examined by gross observation and then by electromyography using a bipolar electode. Secondly, action potential of the regenerating nerves was recorded. Throughout these experiments, the animals were administrated with intravenous drips of succinyl-choline-chloride diluted by 5% dextrose in D/W, and their respirations were controled by a respirator. After these physiological examinations, the peroneal nerves were removed and fixed in formalin for the later histological studies. To evaluate the regenerating axons quantitatively, number of the regenerating axons was counted and histograms were made of Dog No. 17 and Dog No. 38.

A pair of custom-made platinum bipolar electrodes, 0.7 mm in diameter, was used to stimulate the nerve and to record action potential of the nerve. Close attentions were paid to prevent current from spreading, and the electrode was shielded from adjacent tissue by twofold acetate film and polyethylene film. To exclude the influences from reflex pathways through spinal cord or even higher central nervous system, the peroneal nerves were either ligated or sectioned proximal to the site where stimulating electrode was applied. As a rule, the proximal stump of the dissected peroneal nerve was stimulated electrically and the action potential was recorded at the distal stump. On the contrary, antidromic stimulation was done at the distal stump and recording was taken from the proximal stump to confirm distal-proximal conduction of the impulse.

RESULTS

RATS

Macroscopically no definite reactive changes were found around the irradiated grafts with grossly normal appearances. Even microscopically the axons and other structures such as myelin and SCHWANN sheaths were all well maintained even 6 weeks after the operation. In contrast, gross examination of non-irradiated fresh grafts showed marked edema, swelling, petechial hemorrhages and strong adhesions in and around the implants. Histological examination of these untreated specimens revealed marked cellular reactions, degenerative changes with many foreign-body giant cells, massive small round cell infiltration, vascular infiltration with congestion and bleeding of high degree, large amount of swirling fibrosis and collagenization, plasmacytic infiltration, fatty degeneration, and resulting destruction of the nerves.

Dead nerve implants, such as those boiled in water, fixed in alcohol or formalin, provoked less tissue reactions than untreated fresh grafts. Histological examination, however, showed some foreign-body giant cells, moderate amount of small cell infiltration and fibrosis. Neither local application, nor general administration of predonisolone, dexamethasone and cortisone acetate could successfully suppress inflammatory reactions provoked by homologous nerve tissues. Of the general administration of antimetabolites, actinomycin-C could suppress reactions to some extent, but 6-mercaptopurine or methotrexate gave unsatisfactory results.

DOGS

Both ends of the high-voltage electron irradiated nerve grafts were found to be united with the host nerves and a few axons were already seen to penetrate into the graft through the proximal junction 6 days after the operation (Plate 1). At about two months after the operation, the graft looked slightly edematous and was moderately adherent to the adjacent tissues, which, however, could be easily dissected free by blunt disssection (Plate 2). Only in a few cases, some narrowing of the grafts was demonstrated. The adhesions between the arterial tube and the nerve inside were minimum, while the arterial tube adhered moderately to the surrounding tissues. Furthermore, no development of neuromas was grossly observed at either proximal or distal junctures of the grafts when the arterial tube was removed (Plate 3). On histological examination, there were some tissue reactions around the outer surface of the arterial tube, but they scarcely invaded into the inside of the tube (Plate 4). Regenerating axons, which might have spread for all directions independently if there were no splint conducting them straight distally, were seen to run almost parallel to each other through the junctions and the graft with help of the arterial tube (Plate 5). Straying of a few axons through a very tiny slit in the arterial tube could not be avoided though (Plate 6).

In the specimens obtained after 3 weeks of transplantation, only a few regenerating axons were observed, which were scarcely myelinated (Plate 7). On the contrary, moderate numbers of the regenerating axons with considerable myelination were recognized in the specimens obtained at about two months after operation (Plate 5). The axonal concentration and myelination were naturally matured in parallel with elapse of time (Plate 8). Nerve anastomosis with epuineurial sutures formed more or less prominent neuromas at the junctions of the graft and the host nerve with extremely firm adhesions of the graft to the adjacent tissues.

In contrast to the orderly regeneration through the irradiated homografts, strong adhesions developed at the site of grafting and the grafts became swollen markedly with edema when fresh homologous nerve segments without irradiation were transplanted into the gaps of dissected defect in the peroneal nerves of dogs. The regenerating axons could enter only to a little distance into the graft through the proximal junction,but severe fibrotic proliferations and fatty degeneration of the grafts were usually observed to interfere the axons to extend any further (Plate 9).

Previously named antimetabolites were administrated to dogs which had been homografted with irradiated nerve segments. Uneventful postoperative courses were recorded with dogs treated with either actinomycin-C or 6-mercaptopurine, but the dogs to which methotrexate was given developed loss of appetite, emaciation, diarrhea and gastrointestinal bleeding immediately thereafter. Only a few of them could tolerate methotrexate intake at the rate of 0.3 mg/kg for 2 weeks. These experimental animals were sacrificed and autopsied 60 days after operation with the following findings. Methotrexate and 6-mercaptopurine failed to suppress tissue reaction against expectation and a moderate amount of scar tissue was found to be accumulated around the graft. Above all, in 6-mercaptopurine group, the tissue reactions were so variable that the effect of the drug was judged

to be indefinite. Though slight adhesions were found in every dog treated with actinomycin-C, regenerating axons did penetrate through the junction down into the graft in an orderly fashion (Plate 10). Without administration of antimetabolites, the regenerating axons were found to reach to the distance of 3.5～4.5 cm distal to the upper junction in most of the cases at the 60th postoperative day. It is true, however, that all of the antimetabolites used in our experiment demonstrated more or less effects of suppressing the growth of regenerating axons. Only in the actiomycin-C group 3.0～3.5 cm of axonal regeneration was observed, but in the methotrexate and 6-mercaptopurine groups the axonal regeneration was recorded not any longer than 1.5 cm in length.

Histological examinations revealed that the regenerating axons, which passed through the graft down to the distal stump successfully, formed well arranged nerve fascicles. Serial transverse sections of the specimens suggested modality of regeneration of the nerve fibers in the following way. The regenerating axons sprouting from the proximal stump (Plate 11) enter into the grafted nerve segment one after another at the upper junction as if they were scattered all over the cut surface of the segment (Plate 12). Then, they gradually come together to form the nerve fascicles in the graft (Plate 13). Exactly the same pattern takes place at the lower junction (Plate 14) and regenerating axons are finally gathered into the nerve fascicles at the site of the distal stump penetrating throughout the graft (Plate 15). There are significant differences in the diameter of regenerating axons at the early stage of regeneration (Plate 16).

In order to evaluate the nerve regeneration quantitatively, axons were counted mathematically and histogram was made of the regenerating axons of two dogs. Numbers of axons were counted at three places; at the proximal stump, at the middle of the graft and at the distal stump. In Dog No. 17, which was sacrificed on the 203rd postoperative day, axons within the nerve fascicles were 4853 at the proximal stump, 4306 at the middle of the graft and 4003 at the distal stump. In this case, regeneration rate (ratio of axonal count at the distal stump to that at the proximal stump expressed in percentage) of over 80% is recorded, which means that more than 80 % of the regenerating axons could reach the distal stump successfully passing through two junctions. Dog No. 38, which was sacrificed on the 322 nd postoperative day, showed axonal counts of 4880 at the proximal stump, 3016 at the middle of the graft and 3275 at the distal stump, resulting in regeneration rate of about 67 %.

A considerable number of the regenerating axons failed to compose nerve fascicles and were found straying outside of the nerve fascicles in the transverse section of the specimen at the middle of the graft as well as at the level of the distal stump. Close histological study of serial transverse sections, however, revealed that those straying axons outside of the nerve fascicles, although they were directed towards the distal stump, were soon degenerated and finally intermingled into the surrounding scar tissues in a relatively short distance (Plate 13 and 15). In short, those regenerating axons outside of the nerve fascicles, which were observed in some sections by chance, were considered not to participate in ultimate functional regeneration by any means. This is the reason why axons without the nerve fascicles were not counted in axonal count.

Histograms of regenerating axons were carefully studied always being compared with

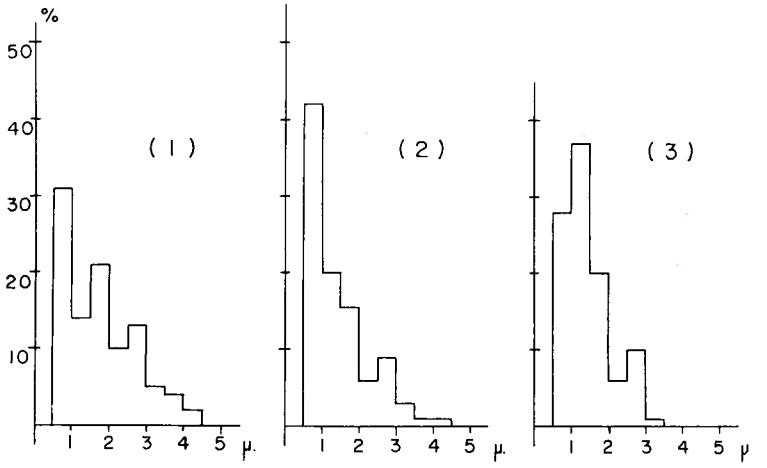

Fig. 2. Histogram. Normal dog.
(1) proximal stump, (2) graft, (3) distal stump.

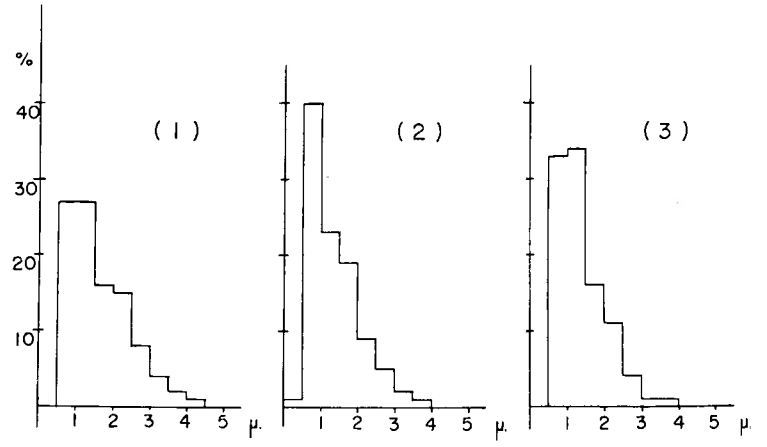

Fig. 3. Histogram. Dog No. 17. 203rd p. o. day.
(1) proximal stump, (2) graft, (3) distal stump.

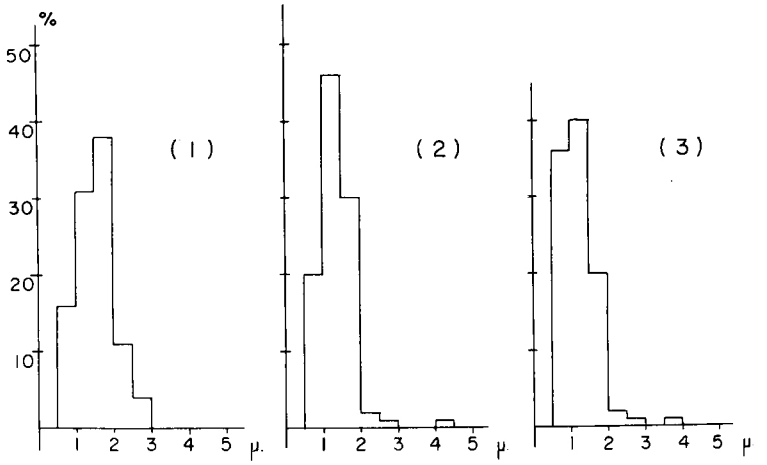

Fig. 4. Histogram. Dog No. 38. 322nd p. o. day.
(1) proximal stump, (2) graft, (3) distal stump.

those of the corresponding levels of normal nerves of the same name. Histograms of normal nerve demonstrated two peaks, one of which was comprised of large axons (Fig 2). On the other hand, regenerating nerves displayed only a peak because of the lack of large axons (Fig. 3 and 4).

Histological study of the regenerating nerve focused on degree of myelination revealed that the regenerating axons within the nerve fascicles were well myelinated even at the level of the distal stump, though the thickness of myelin sheath showed some varieties. In contrast, regenerating axons outside of the nerve fascicles were mostly not myelinated, though some of them were poorly done (Plate 17).

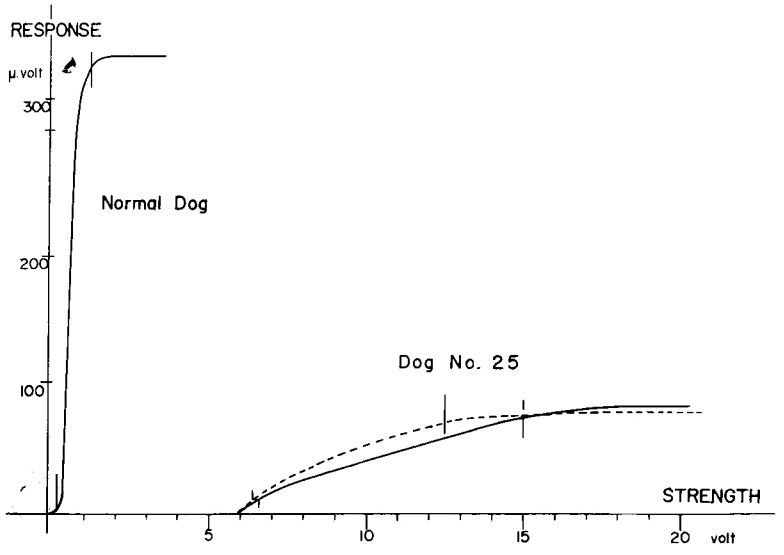

Fig. 5. Stimulus-response curve. Dog No. 25. 202nd p. o. day.
-------- after potassium-ion application.

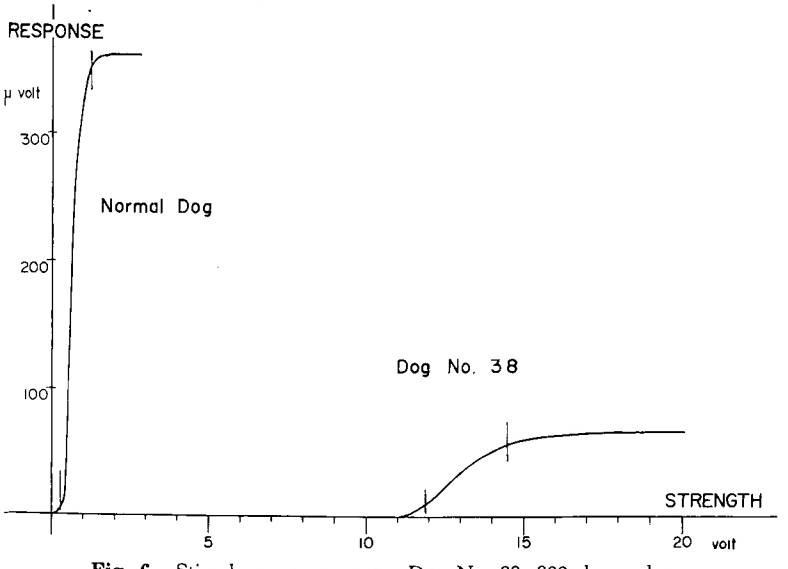

Fig. 6. Stimulus-response curve. Dog No. 38. 322nd p.o. day.

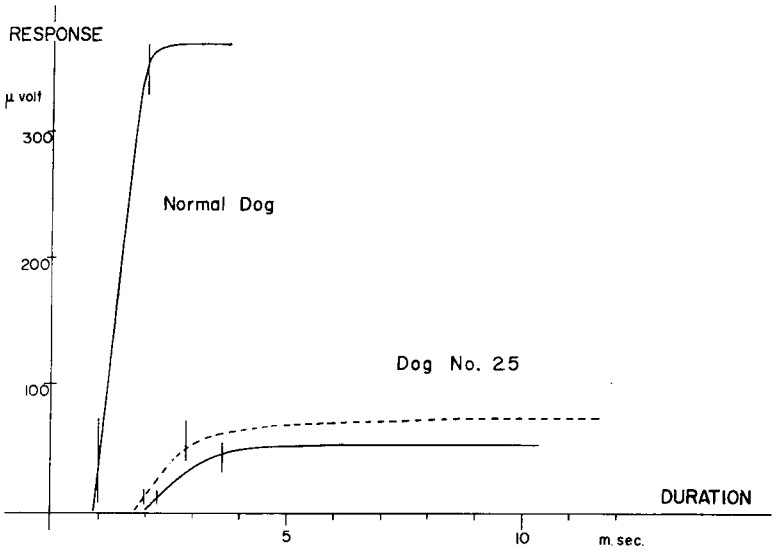

Fig. 7. Refractory time. Dog No. 25. 202nd p. o. day.
..... after potassium-ion application.

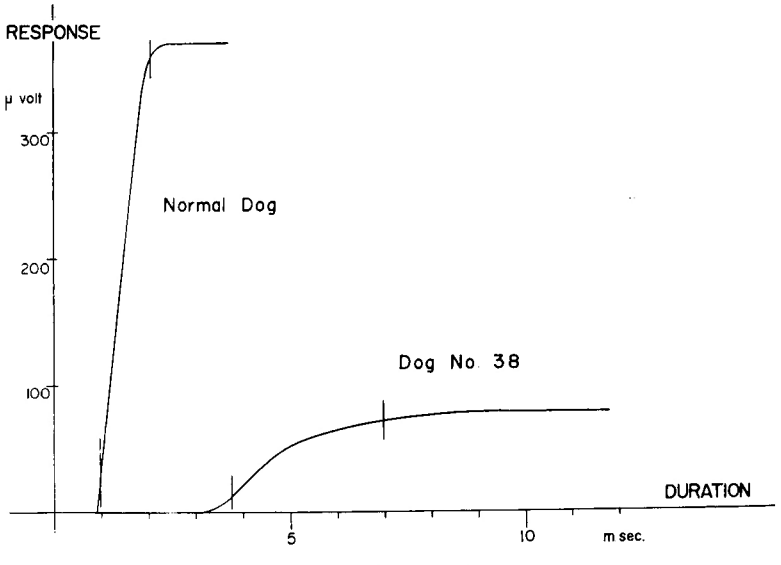

Fig. 8. Refractory time. Dog No. 38. 322nd p.o. day.

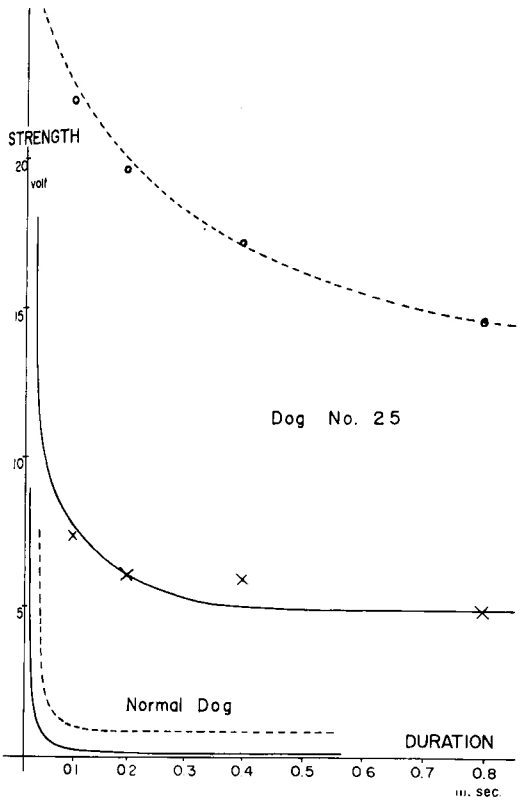

Fig. 9. Strength-duration curve. Dog No. 25. 202nd p. o. day.
..... supramaximal stimulation,
——— threshold of stimulation.

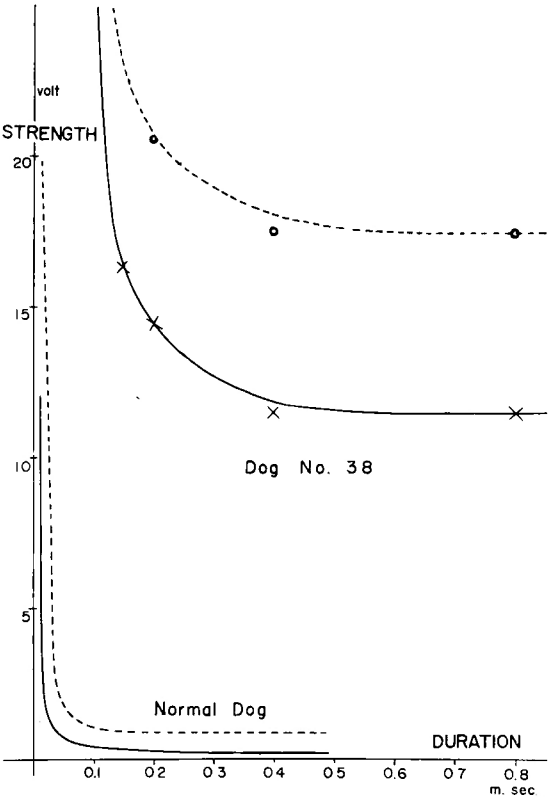

Fig. 10. Strength-duration curve. Dog No. 38. 322nd p. o. day.
..... supramaximal stimulation,
——— threshold of stimulation.

Electrophysiological study was made in twelve cases of long term observation and conductiveness of evoked action potential was noted in all cases (Table 1). However, elevated threshold of stimulation, low voltage of evoked action potential, elongated refractory time and retarded conduction velocity were demonstrated. In Dog No. 25, for example, threshold of stimulation was about 12 volts, when normal nerve showed threshold of below 1 volt. Evoked action potential became one-fifth, namely fell down to about 70 μ volts from the value of 350 μ volts recorded in cases of control dogs (Fig. 5 and 6). Local application of potassium-ion solution to stimulate electrode lowered supramaximal stimulation (Fig. 5). Refractory time was measured by use of double shock technique. Although control nerve had absolute and relative refractory times of about 1m sec and 2m sec respectively, those of regenerating nerve were elongated to 2.2 m sec and 3.7 m sec in Dog No. 25, and to 4 m sec and 7m sec in Dog No. 38, respectively (Fig. 7 and 8). Refractory time was also shortened by local application of potassium-ion solution (Fig. 7). Though conduction velocity of control nerves was 50~55 m/sec, that of experimental dogs was nearly half in comparison with the control nerve; in dogs examined about 6 months after the operation, 20~40 m/sec, and in Dog No. 38, 27 m/sec respectively.

These action potentials were evoked and transmitted either when the proximal stump was stimulated and the graft or the distal stump was induced or, vice versa, when the distal stump was stimulated and the proximal stump was induced. Ligation or sectioning of the proximal and/or the distal segment of the nerve ruled out the possibility of any other pathway influencing the action potential recording. In 5 dogs out of 12 cases of long term observation, stimulation of the nerve resulted in a vigorous contraction of the dorsiflexors of the feet.

DISCUSSION

MEEKER and GROSS[26] studied high-voltage cathode ray irradiation from the view points of sterilizing power against contaminated vascular segments and its influence to the vascular wall such as denaturating, degrading or depolimerizing of the organic tissues. They found that satisfactory sterilization effect was obtained in the irradiation range between 2.0 million and nearly 2.5 million REP at −55°C. In their paper, it is said that very little or almost no tissue damage was inflicted under the above described condition. At least 1.5 million REP of irradiation was necessitated in order to secure sterility with high degree of regularity, but doses at 3.0 million REP or above produced severe and irreversible changes in many of the grafts. TURNER and his associates[44] stated that irradiation of tissue with 2 million REP by a VAN DE GRAAFF generator was a successful method of tissue sterilization without producing foreign-body reactions which usually occurred otherwise. ASHLEY et al. reported successful homologous tendon grafts in chickens with help of cathode ray irradiation.

The informations gained from these basic studies encouraged MARMOR to apply the electron irradiation for nerve transplantation as a method of reducing foreign-body reactions and sterilizing homologous nerve grafts[21]. The following facts were previously pointed out in our laboratory by NAOKI KAGEYAMA, SHŌJI NAKAJIMA and me[12][13] in connection with studies on immunosuppressive action of high-voltage cathode ray irradiation in rats and dogs: Irradiating the grafted nerve segment prior to implantation gave far superior immunosuppressive effect than any other possible methods such as freezing in deep freezer, boiling in hot water, fixing in alcohol or formalin, and local and/or general administration of andrenocortical hormones or antimetabolites. Electron irradiated homograft did not develop any serious adhesions clinically and histological study of the specimen revealed very slight foreign-body reactions such as mild cell infiltration and minimum vascularization. No evidence of hemorrhage, fibrosis or foreign-body giant cell was proved microscopically. The original structure of the nerve was well maintained even 6 weeks after operation upon microscopic examination.

BUNNELL[2] reviewed the work on hetero-, homo- and auto-grafts and concluded that long nerve homografts were unsuccessful even in animal experimentation, because the immunity of the host destroyed the nerve graft befor the axons could have penetrated through the graft if it had been longer than 2 cm in length. He stated, "It is a race between the growth of the axons through the graft and the development of active immunity in the host".

Recently many scholars have reported successful organ homotransplantation by various

immunosuppressive measures. It can be said, as far as peripheral nerve transplantation is concerned, that preoperative treatment of the grafts seems to be most important practically. General administration of adrenocortical hormones failed to suppress reactions when they were applied to the nerve homotransplantation, and even development of serious complications was observed when antimetabolites had been administrated.

It is true that high-voltage electron irradiated homograft does not produce any severe reactions in and around the grafts in dogs and a good many regenerating axons successfully reach the distal nerve stump penetrating through the graft and they keep growing on with functional recovery. However it has not yet been clearly explained what kind of reaction, and to what extent, has been caused in the grafted nerve tissue by electron irradiation, that has made it possible to suppress the development of active immunity in the host so effectively. Irradiation of several million REP on the tissue at room temperature is said[26] to produce drastic alterations in enzymic systems and organic composition. PATT[28] reviewed acute effects given by radiation of high energy on mammalian systems, and stated that while all of the ionizing radiations produced more or less similar biological effects, their efficiency could vary considerably. This depended apparently upon the absolute amount of energy to be absorbed in space and time and also upon its distribution in the organ. Metabolic derangements induced by high energy radiation are said to be resulted from a primary attack on enzymic systems and other biochemical mechanisms, or as a consequence of cellular breakdown and the various physiological ramifications. LUZZIO[19] described that the immunoelectrophoretic patterns of human serum γ-globulin, subjected to 3.66～5.49 megaroentgens (MR) of X-rays, had exhibited increase in slow moving components, which had been absent in the original human serum γ-globulin. WACHSMANN and his associate[45] reported the effects obtained by irradiating plants and animals with doses up to 12 MR. They assumed the presence of "radiotoxine" from the facts that radiation effects were found to be spread widely in plants even beyond the irradiated area, and in animals even death occurred when the radiation was limited to the organs which were believed to be of no importance for their lives. HARDY[8] stated that the physical or X-ray energy was, as the velocity and mass of the rays being dissipated, transformed into chemical energy and consequently chemical changes were caused in the target materials. Thus, the electron composition being altered and the structure of an atom being changed, a substance of different chemical properties would have been produced. Tissue culture study of the irradiated nerve tissue attempted at our laboratory with technical help by YUTAKA TADA failed to demonstrate any outgrowth. It is of course dangerous to conclude merely from this result that the irradiated nerve tissue was not alive. However, as MEEKER and GROSS stated that sterilization is almost always achieved by 2.0 million REP irradiation, it seems to be reasonable to suppose that considerable changes must have taken place in enzymic systems of chromosomes and proteins that comprise cell body.

When the cut nerve is reunited, SCHWANN cells are believed[31] to hypertrophy, increase in number and then fill the endoneurium with their protoplasm. Along with the vascularization at the distal segment of the nerve, the nerve fibers run after the ingrowing blood vessels. Later, axons increase in diameter and are gradually myelinated[10]. When a dead nerve segment is homografted, primary union between the graft and the host nerve

takes place at first, and then the newly developing nerve fibers travel through the SCHWANN tube of the graft from the proximal stump crossing over the upper junction down to the distal stump, provided that the grafted segment keeps maintaining a channel to pass through without causing critical foreign-body reactions. They may begin to myelinate thereafter. By high-voltage electron irradiation, the graft is probably kept insoluble, i. e., the external cell form may be kept unchanged and channel of SCHWANN tube may be hold open with the tissue constituents being not drastically destroyed. This is probably why irradiated nerve graft does not allow active immunity to develop in the host and a state of immunological tolerance is produced in cases of irradiated homografting.

For many years, epineurial suture has been the most widely and popularly accepted technique for nerve anastomoses. In spite of a device to resect the epineurium slightly longer than the cut end of the nerve, this sort of conventional suture technique, however, essentially entangles some nerve fibers in the suture, no matter how carefully the operation is performed. In addition, it is technically difficult to avoid knuckling of the nerve fascicles and axial rotation of some degree. Thus mentioned disadvantages of the conventional suture technique cause malalignment of the groups of sensory and motor fibers between the two stumps afterwards. Moreover, all suture materials now available are more or less foreign to the living tissues and they naturally cause tissue reactions[5]. Because of these unfavorable but unavoidable faults, fibrosis begins in and out of the nerve, which results in formation of considerably sized neuroma and severe adhesions between the graft and the surrounding tissues.

WEISS[51] has studied and summarized the materials and methods used for nerve wrapping and tubulization. He stated in his paper that tubulation technique should not be considered merely as providing tubes for regenerating axons, but it should be properly evaluated rather as a method for providing a well-oriented ultrastructure for the regenerating axons. KLINE[14], wrapping around peroneal nerves in adult chimpanzees with processed bovine flexor-tendon collagen, found that the wrapped nerves had less disorganization at the site of repair and axonal carry-through was more orderly and concentrated than the unwrapped control nerves. TAKETOMO[42] and WATANABE[46][47] in our clinic performed sutureless reunion of a severed nerve with success by tubulation technique using an arterial tube fixed and preserved in 70 % alcohol. By their technique, they said, a firm link could be established between the nerve ends, and straying escape of regenerating axons and formation of a neuroma could be satisfactorily prevented. In the present experiment, irradiated arteries were used as tubes, and the results thus obtained were satisfactory. In two out of 14 long term observations, separation at the lower junction was noted upon exploration. These two dogs were operated on in early stage of the experiment when the operative technique was rather poor and unstable. In our opinion, the nerve segment to be grafted should always be longer than defect in length by all means in order to obtain satisfactory result.

As being previously described, once irradiated, arterial tube is usually free from serious tissue reactions when it is implanted, and therefore, extracellular fluid, which supply nutrition to the regenerating nerve, can freely and amply pass through the implanted arterial wall and flow through the space between the arterial tube and the grafted nerve

segment existing in it. This seems to be one of the most important reasons why the excellent results were gained in this series of the present experiment. CAMPBELL and his associates[3)4)] pointed out that free diffusion of nutrient extracellular fluid through the covering is necessary for migration and proliferation of the neuronal elements at the proximal nerve stump. From those standpoints of view, together with other advantages confirmed by various authors[9)14)42)46)47)49)50)51)], tubulation made of a short inert arterial segment is assumed to be one of most suitable methods for nerve anastomoses. As said in a proverb, "Too much is as bad as too little", it would be of course undesirable to wrap the grafted nerve segment with a too long arterial tube or with tube made of other brittle materials. It goes without saying that nerve anastomosis without any foreign materials is naturally ideal. Plasma clot method of nerve suture, initiated by YOUNG and MEDAWAR[53)] and improved by TARLOV[38)39)40)41)] later on was not perfect though. It had even ultimate shortcoming that the technique could be applied only where there was no tension, and that with minimal tension the reunion was easily separated at early stage. Funicular suture or funicular exclusion technique is theoretically rational from the functional standpoint[37)], but either can never be applied correctly without profound and detailed knowledge of the intraneural topography of the major peripheral nerves, and moreover there is no experimental proof that the use of such complicated techniques will improve functional recovery any better[6)].

Properly applied tubulation technique prevents fibroblastic invasion from the surrounding tissues almost completely, but it is not capable of preventing the proliferation of fibroblasts from the cut ends of the host nerve and consequent slight disorganization of the regenerating axons is usually found by microscopic examination. General administration of antimetabolites, especially actinomycin-C, revealed considerable effectiveness in suppressing fibroblastic proliferation from the nerve ends. Regardless of the kinds, those antimetabolites, however, suppressed not only the tissue reaction but also outgrowing vitality of the regenerating axons to some extent. As being previously mentioned, successful recovery of function was attained in the present experiment by a simple method of irradiating homografted nerve segments prior to implantation, and administration of so-called antimetabolites was not necessitated for the purpose of functional recovery. It was proved to be even undesirable to administrate antimetabolites. SEDDON[35)] also recognized no justification for employment of the drastic measure required to suppress the homograft reaction in the nerve grafting.

Histological study of the graft and the distal stump where functional recovery had been demonstrated, showed that there were grossly two groups of regenerating axons; one consisting of the axons in the nerve fascicles and the other straying out of them. In regard to the fiber connection with peripheral end-organs, these two groups were entirely different. The regenerating axons gathered in the nerve fascicles were well myelinated, and were connected successfully with peripheral end-organs. These axons take leadership in functional recovery of the nerve. In contrast, regenerating axons outside the nerve fascicles rarely myelinated and disappear in the course of a relatively short distance with fatty degeneration. These axons would not contribute in functional recovery.

Neuroregeneration is considered to be the result of ameboid extension of the severed

tips of the axons. Endoplasm, being synthetized in the cell body, flows towards the periphery in continuous proximo-distal axoplasmic flow and is deposited at the fiber-tip[48)52)]. Elongation of the axis cylinders is resulted by the squeeze of axoplasm into the region of the pseudopods which extend distally with ameboid movement[17)]. At the proximal stump, many pseudopod-like structures are extended from a single nerve fiber during the process of regeneration, and therefore, many irregularly disposed small axons, which are usually 3～5 times as many in number as at the proximal stump, are counted in the transverse cut surface both at upper and lower junctions. According to our opinion, only those nerve fibers that have successfully entered into the SCHWANN tube do grow distalwards straightly forming nerve fascicles, and those which have failed to find their way in the endoneural tubes run only for a short distance towards the periphery without any fascicle formation and they are finally absorbed with degeneration. This is the reason why only the regenerating axons existing in the nerve fascicles are counted as the effectively regenerating axons. 67% and above 80% of regeneration rates were recorded with two dogs of long term observation, being based upon axonal counts. Regeneration rate seems to indicate to what extent that the SCHWANN tubes exist with function providing routes for regenerating axons to go through. Such high regeneration rates recorded in two cases of our present experiment suggest that most of the original tubes were filled with newly regenerating axons effectively.

The specimens were fixed in 10% neutral formalin, imbedded in paraffin and silver-impregnated after HOLMES' technique. Artificial shrinkage of the specimens naturally occurred during the process and consequently axonal diameters on histogram became by far smaller than they should have been otherwise. Judging from the fact that the conduction velocity of normal nerve is 50～55 m/sec, the axons should have diameter of 6-11 μ[27)]. However, even relatively large axons, composing the second peak in histogram of control nerves, have average diameter of only 2.5～3 μ. It can be said, therefore, that the specimens have shrunk to one-third of original size during the process. Nevertheless, if all of the regenerating axons have shrunk with uniform rate —— and perhaps this is precisely what has happened—present data can be accepted sufficiently and safely for the comparative study. THULIN[43)] and JENKINS et al.[11)] have found that the regeneration rates of the fibers of different sizes were approximately the same. The diameter of the regenerating nerve fibers, however, changes depending not only upon the original size of the fiber but also upon the duration of regeneration.

Development of clinical signs such as lameness or crippling, foot-drop, weakness, ulceration, atrophy, loss of hair and deformity of nails has been reported after crushing, sectioning, suturing, nerve-grafting and crossed anastomosis of sciatic nerves in experimental animals. It is also said, that the discrepancy between the experimental findings and their clinical applications is partly due to the fact that in the clinical applications, the recovery of function is chiefly evaluated by clinical observations but, in contrast, the evaluation of experimental results is usually based upon histological and/or physiological ones. In experimental animals, however, it is difficult to evaluate the recovery of nerve function only by clinical observations because loss of some functions seems to be usually compensated satisfactorily.

In order to judge the degree of functional recovery more correctly after nerve homotransplantation, dorsiflexion of the foot by electric stimulation was observed and action potentials of the nerve were also recorded. Action potential tells directly the true condition of the nerve itself eliminating many factors which may have influenced. Current spread was cautiously prevented by strictly shielding the surrounding tissues from the stimulating and recording electrodes. To rule out the influence of any other nerve pathways, the nerve was ligated or sectioned proximal and/or distal to the graft if needed. The influences of electromyogram were satisfactorily eliminated by blocking the neuromuscular junctions with intravenous dripping of succinyl-choline-chloride. The nerve proximal to the graft was usually stimulated and the record was taken at the distal stump, and the antidromic stimulation was also applied. It is difficult to explain the marked elevation of the threshold of stimulation and the marked decrease in voltage of action potentials recorded, only by morphological fact of moderate decrease in the axonal count which really verifies some decrease of regenerating axons in number histologically. It can, however, be understood in the following way: In the first place, the effectiveness of stimulation and conduction was diminished by the surrounding scar tissues. And secondly, as shown on histogram, large axons with strong electromotive force were missed in the regenerating nerves. The refractory periods correspond the time interval during which membrane potential of the nerve fiber, once depolarized by conditioning stimulation, is restored to a certain level and gets ready to respond on the test stimulation. They are regarded as indicative of blood supply and excitability of the nerve. Because potassium-ion solution is able to accelerate the restoration of membrane potential, the refractory periods are shortened when potassium-ion solution is applied to the stimulating electrode, and this is the characteristic of the normal nerve. POLLACK et al.[30] stated that the appearance of discontinuity in a strength-duration curve was a more accurate indication of recovery than a diminution of the chronaxie. However, neither discontinuity in the strength-duration curve, nor constancy of diminution of the chronaxie was observed in Dog No. 17 and Dog No. 38 (Fig. 9 and 10). As the regenerating nerve had few large axons with faster conduction velocity, conduction velocity might have been retarded in experimental animals. Conduction velocity of several dogs of approximately 200 postoperative days were measured to be 20-40 m/sec. These figures, together with the thickness of axons and the maturation of myelination on histological study, indicate that the regenerating axons have been considerably ripe 200 days of homografting with irradiated nerve. However, the fact that only three cases out of eleven, which had been observed for nearly 200 days postoperatively, demonstrated dorsiflexion of the foot on electric stimulation suggests that regenerating axons gain the maximal thickness within 200 postoperative days, though functional connection with peripheral end-organs are not yet completely re-established by then.

Because of the uniformly excellent results in the animal experiments, two cases of clinical use of the same technique were lately carried out.

PRELIMINARY REPORT OF TWO CLINICAL CASES

CASE 1. O. H., a 56-year-old female, with a history of total extirpation of a huge neuroma from the left common peroneal nerve two weeks prior to the admission to our

clinic. According to what the surgeon and patient mentioned, the tumor was so large in size and so firmly adhered to the main trunk of the nerve as well as to the surrounding tissues that the intracapsular enucleation without giving serious damage to the nerve was not technically possible then. It was therefore extirpated together with the main nerve trunk for the length of a few centimeters, leaving complete loss of motor and sensory functions postoperatively. She was admitted to our clinic for nerve grafting. A portion of the lumbar nerve was resected from a fresh cadaver and was prepared for the graft. A portion of the femoral artery was also resected at the same time and was prepared for making the arterial tube. These two specimens were treated properly with irradiation and freezing prior to implantation. Upon exploration, neuromas were found at both ends of the common peroneal nerve, which were surgically excised leaving a defect of about 10 cm. A nerve graft of 13 cm in length was sutured in place by the tubulation technique. 13 months after operation, peripheral areas of numbness were noted to be markedly decreased, though no evidence of recovery in motor function was obvious.

CASE 2. T. M., an 18-year-old boy with a history of ulnar nerve laceration at the point of distal two-thirds of the right forearm, which was sutured primarily by a surgeon 3 months prior to the admission to our clinic. No signs of functional recovery, either motor or sensory, were observed after the primary operation. The second operation was therefore undertaken by us. A black silk suture was found to strangle the nerve at the area of repair upon exploration. After resecting the junction together with the neuroma, a 3.5 cm nerve graft was inserted to fill up the operative defect. Histological study of the resected specimen revealed no axonal carry-through of the junction. Functional recovery of adduction of the 5 th finger was proved 4 months after surgery.

Various techniques for repair of peripheral nerves have been reported to be successful and promissing in the experimental laboratories, but their clinical application to the human beings have been often proved to be unfavorable because of the difference of species in response to peripheral nerve injuries[34]. Being encouraged by the satisfactory results which I and my co-operaters had obtained so far in our laboratory, I have tried electron irradiated nerve homograft clinically to the selected patients with large defects, for whom nothing but grafting was indicated for possible functional recovery. Fortunately favorable and satisfactory postoperative clinical courses have been observed with our two cases of initial trial. As STERN once stated[36], application of homografting nerve tissue in the human being was limited simply because freshly amputated limbs were only the available source of obtaining the grafts. With our new technique of preserving the grafts, what STERN had mentioned became not true any longer. High-voltage electron irradiation now permits obtaining the grafts even from fresh cadavers, and preservation of the grafts in deep freezer at the temperature of below -25 degrees centigrade allows to keep them for a long period of time effectively without any physiological damages.

SUMMARY

It was previously reported that peripheral nerve of rats and dogs could be successfully homografted if they had been irradiated with two million REP of electron beam for the purpose of suppressing the immune reaction. Histological and electrophysiological analyses are hereby attempted on fourteen cases which have been successfully kept alive for long term observation. In addition, availability of vascular tubulation technique to nerve anastomoses and importance of general immune suppressive treatments in cases of nerve homografting are discussed.

In twelve successful cases out of fourteen, which had been followed up for more than 180 days after operation, regenerating axons were found to penetrate through the graft down into the distal stump mostly with fascicle formation. Axonal count at the proximal stump, the grafted portion and the distal stump perfomed with two dogs, which were sacrificed at 203 rd and 322 nd postoperative days, proved more than 80% and 67% of regeneration respectively. On the histogram, the regenerating axons were somehow smaller in diameter in comparison with corresponding portion of the normal nerve fibers. At this stage, namely, approximately 200 days after surgery, myelination of regenerating axons was fairly well accomplished but moderate variation in the thickness of myelin sheath, i. e., in the diameter of endoneural tube was always noted. Some of the regenerating axons which failed to develop through the grafted fascicles were seldom myelinated, and were spread irregularly towards distal direction for a short distance. These scattered fibers disappeared soon thereafter being displaced by scar tissue. From this fact, these non-myelinated nerve fibers appear not to take any role in functional regeneration of the nervous system.

Electrophysiological examination of the regenerating axons demonstrated elevated threshold of stimulation, elongated refractory time and slowed conduction velocity in comparison with normal nerve of the same name. These changes could be partly compensated when potassium-ion solution was locally added to the electrode ; supramaximal stimulation being lowered and relative refractory time being shortened. The above mentioned fact suggests that the regenerating axons have wide range of electrosensitivity.

Arterial tubulation technique applied on nerve anastomoses effectively prevented scar tissue invading into the line of anastomoses and, moreover, the tube led the regenerating nerve fibers directly down to the distal. No macroscopic evidence of neuroma formation was ever proved grossly in any of present cases.

Methotrexate, actinomycin-C and 6-mercaptopurine were tested for the purpose of suppressing microscopic formation of neuroma with acceptable results only in cases of actinomycin-C administration. The other agents seemed to be non-effective.

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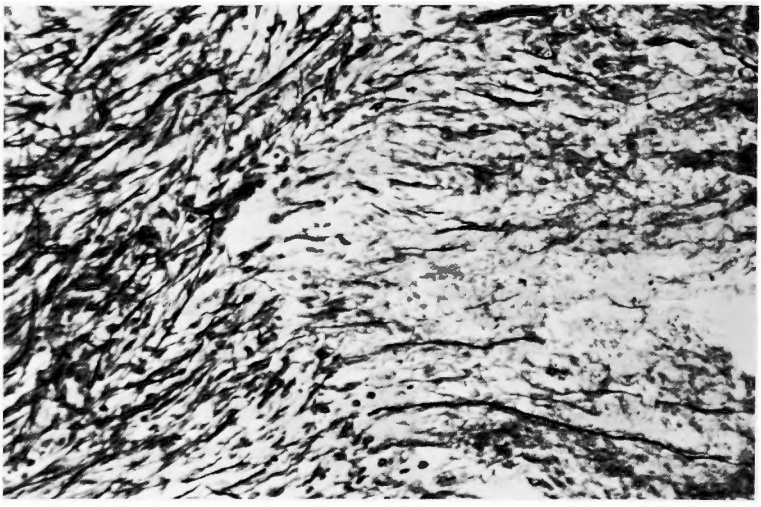

Plate 1. Dog No. 57. 6th p.o. day. A few regenerating axons already penetrate the arc-shaped line of suture. left : proximal, right : distal.
(×100 Holmes' strin)

Plate 2. Dog No. 44. 60th p.o. day. Gross appearance of the graft and the arterial tube. Slight edema of the graft portion with minimal surrounding tissue reactions. Nylon and silk sutures are seen. left : proximal, right : distal.

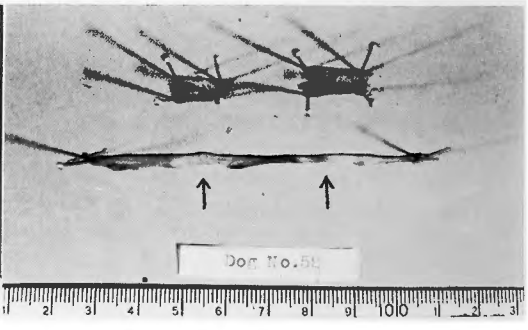

Plate 3. Dog No. 52. 63rd p.o. day. No evidence of gross neuroma formation. Arrows point to the line of suture. left : distal, right : proximal.

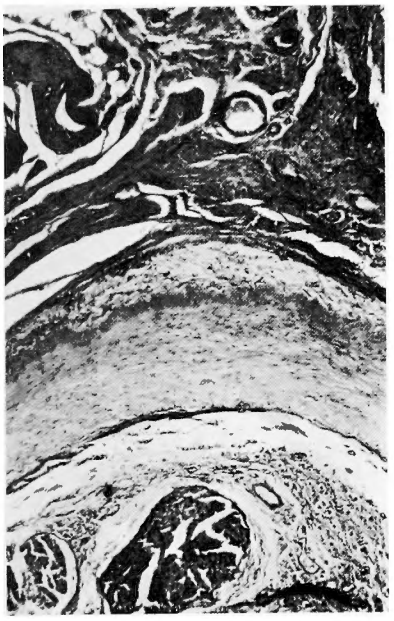

Plate 4. Dog No. 45. 62nd p. o. day. Severe fibrosis on the outside (top) of the arterial tube, which is due to wound infection, is seen not invading the arterial wall. (×40 H-E)

Plate 5. Dog No. 74. 64th p. o. day. Beads-like appearance of regenerating axons. Slight axonal disorganization. Myelination is not yet complete. (×100 Holmes' stain)

Plate 6. Dog No. 36. 14th p. o. day. Straying escape of the regenerating axons through the tiny slit in the arterial tube. bottum : inside, top : outside, of the arterial tube. (×100 Holmes' stain)

Plate 7. Dog No. 37. 21st p. o. day. Non-myelinated regenerating axons penetrating the graft. (×100 Holmes' stain)

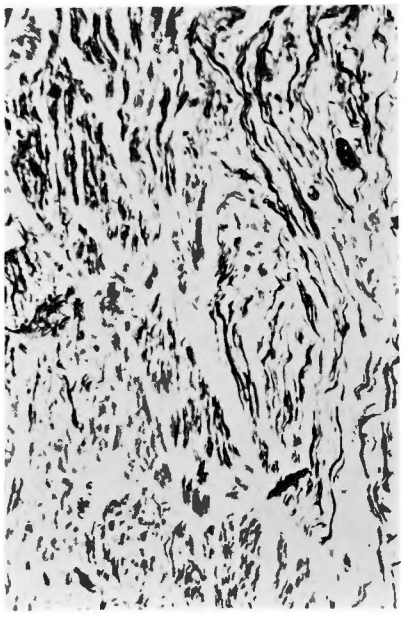

Plate 8. Dog No. 38. 322nd p. o. day. Highly concentrated and well-myelinated regenerating axons carrying through the graft. Slight axonal disorganization. (×100 Holmes' stain)

Plate 9. Dog No. 55. 29th p. o. day. Fresh graft. Severe fibrotic reactions and fatty degeneration of the graft. (×100 H-E)

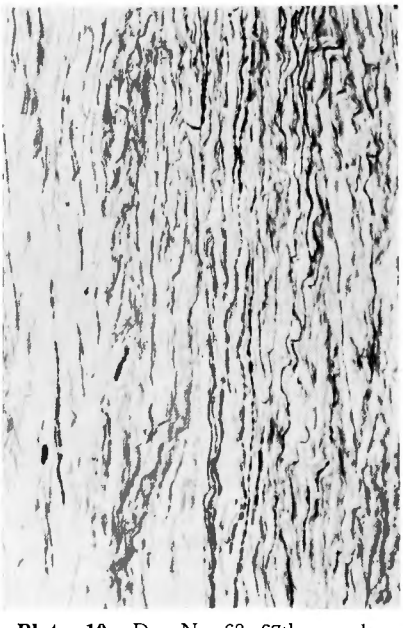

Plate 10. Dog No. 68. 67th p. o. day. Actinomycin-C administration. Well-oriented regenerating axons. (×100 Holmes' stain)

Plate 11. Dog No. 17. 203rd p. o. day. Proximal stump. Fascicles of the host never. (×100 Holmes' stain)

Plate 12. Dog No. 17. 203rd p.o. day. Upper junction. Irregularly disposed small axons filling intra-arterial-tubular space. top : arterial wall.
(×100 Holmes' stain)

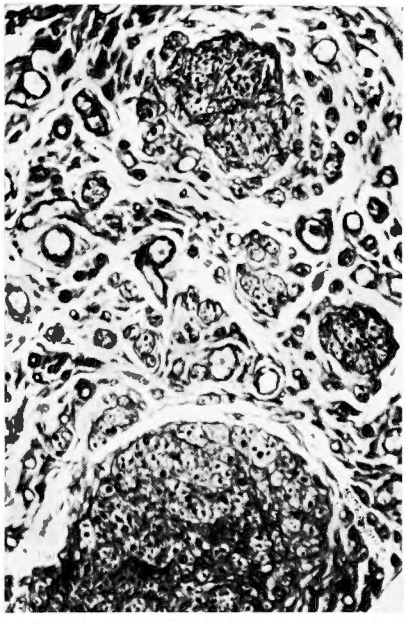

Plate 13. Dog No. 17. 203rd p.o. day. Graft. Most of the regenerating axons are gathering with fascicles formation. Others are sporadically scattered outside the fascicles, some of which are already fatty-degenerated.
(×100 Holmes' stain)

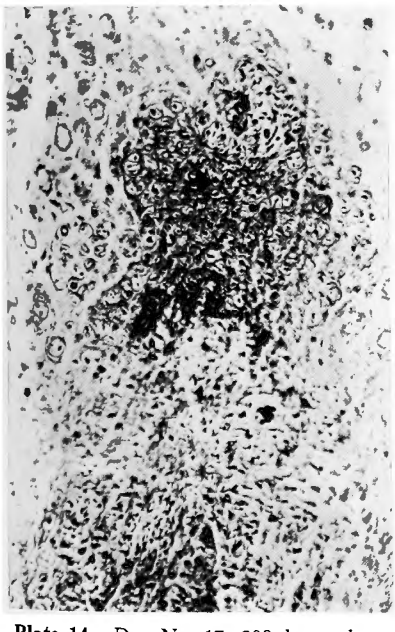

Plate 14. Dog No. 17. 203rd p. o. day. Lower junction. Once more scattered regenerating axons.
(×100 Holmes' stain)

Plate 15. Dog No. 17. 203rd p. o. day. Distal stump. Well-organized fascicles formation. Straying axons are completely degenerated and disappear in this level. (×100 Holmes' stain)

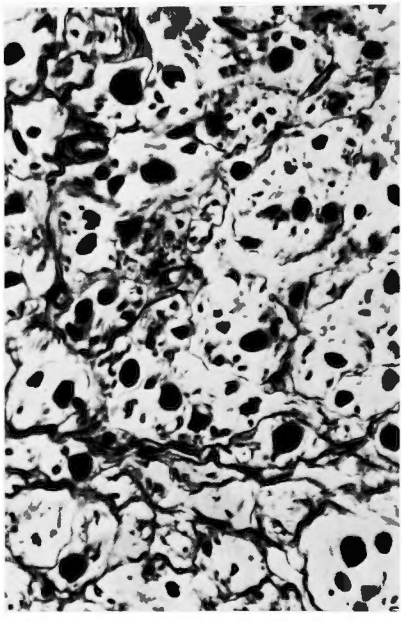

Plate 16. Dog No. 17. 203rd p. o. day. Graft. High-power magnification of the newly formed fascicles in Plate 13. Marked difference in diameter of regenerating axons.
(×400 Holmes' stain)

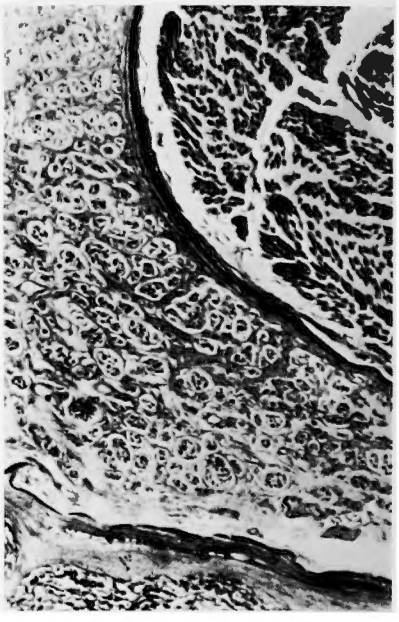

Plate 17. Dog No. 22. 180th p. o. day. Well-myelinated and concentrated regenerating axons within the fascicles (top-right), in contrast to the scarcely myelinated axons scattering outside the fascicles. bottum : arterial tube.
(×100 Holmes' stain)

和文抄録

高圧電子線照射による末梢神経同種移植

京都大学医学部脳神経外科学教室（指導：半田　肇教授）

池　田　公　行

末梢神経損傷の修復は現在に至る迄外科治療の困難な問題の一つである．多種多様な手術方法ないし神経移植の方法が試みられ，動物実験ではある程度の成功が認められているが，その臨床的応用は必ずしも容易ではない．我々は先に，ラッテおよび犬の末梢神経にドライアイスアルコール浴中で200万REP の電子線照射を行なつて免疫反応を抑制すると，同種移植が成功する事実を報告した[7)12)13)]．

本文は雑種成犬に照射神経片を同種移植し長期観察を行なつた14例の神経再生を，組織学的および電気生理学的に検討した結果を報告する．併せて神経縫合法としての動脈管外套法の意義，および末梢神経同種移植における全身的免疫抑制療法の意義について述べた．また最近本法を臨床的に応用した２例の術後経過に触れた．

1) 術後180日以上観察した14例中，成功例12例では，再生神経線維は神経線維束を形成して移植神経片を貫通し，遠位神経端に達していた．

2) 術後203日目および322日目の各１例で夫々80％以上および67％の神経再生率が認められた．

3) 術後約200日目には再生神経線維の直径は全体としてやや細く，且つ個々の太さにはかなりのばらつきが認められた．この時期には髄鞘形成はかなり成熟していた．

4) 再生神経では，電気生理学的に刺激閾値の上昇，不応期の延長および伝達速度の遅延が認められたが，これらはカリウム溶液の局所使用により一部正常化された．

5) 動脈管外套法により神経縫合を行なうと，外部から縫合部への瘢痕組織の侵入を阻止し，同時に再生神経線維を真直ぐに末梢へ導く副木となり，肉眼的に認め得る神経腫が形成されなかつ．

6) Methotrexate, actinomycin-C および 6-mercaptopurine を全身的に投与した結果，actinomycin-C は使用に耐えるが，いづれも同時に神経再生を抑制する効果を示した．